Exhibit 1

Expression of the G-Protein–Coupled Receptor BLR1 Defines Mature, Recirculating B Cells and a Subset of T-Helper Memory Cells

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The G-protein–coupled receptor BLR1 related to receptors for chemokines and neuropeptides has been identified as the first lymphocyte-specific member of the gene family characterized by seven transmembrane-spanning regions. Using a high-affinity anti-BLR1 monoclonal antibody (MoAb) and three-color flow cytometry it is shown that BLR1 expression on peripheral blood cells is limited to B cells and to a subset of CD4+ (14%) and CD8+ (2%) lymphocytes. T cells expressing BLR1 were positive for CD45R0, were negative for interleukin-2 receptors, show high levels of CD44, and show low levels of L-selectin. The majority of CD4+ cells originating from secondary lymphatic tissue, but none of cord blood-derived T cells, express BLR1. These observa-tions suggest that BLR1 is a marker for memory T cells. Furthermore, BLR1 expression was detected on all CD19+ peripheral or tonsillar B lymphocytes, but only on a fraction of cord blood cells and bone marrow cells expressing CD19, sIgM, or sIgD. Interestingly, activation of both mature B and T cells by CD40 MoAb and CD3 MoAb, respectively, led to complete downregulation of BLR1. These data suggest that the G-protein–coupled receptor BLR1 is involved in func-tional control of mature recirculating B cells and T-helper memory cells participating in cell migration and cell activa-tion.

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IMMUNOLOGIC memory is one of the key features of the immune system of higher vertebrates and provides a rapid and excellent response of an organism to secondary encounters with pathogens. Despite recent evidence in clon-ing and analyzing factors involved in differentiation and maturation of both B and T cells, there is still considerable controversy about the phenotype of immunologic memory cells.[1-9] B cells are rapidly produced by hematopoietic stem cells of the bone marrow and the fetal spleen. However, most of these cells die soon after exit from the marrow and those that survive are considered to reconstitute the red pulp and T-cell–rich zones of the spleen or, in general, to popu-late secondary lymphatic tissues.[10-12] During the passage from marrow to secondary lymphatic tissue, B cells are se-lected by ligands as they differ from bone marrow cells in V gene expression.[13,14] These cells are considered to be naive and, together with memory cells, they form the stable periph-eral B-cell pool. This pool consists of B cells recirculating between lymphatic tissues and of sessile cells of the marginal zone (MZ).[15] After primary contact with an antigen, naive B cells of the T-cell–rich zone are activated and then migrate to follicles where they give rise to the formation of germinal centres. Here, B cells undergo two important changes: hyper-mutation and selection of high-affinity B cells and generation of memory B cells. Once having entered the follicle, B blasts grow exponentially and populate the follicular dentritic cell (FDC) network, eventually leading to development of the typical polarized shape of the germinal center. Although still in rapid cell cycle, the centroblasts, unlike their progenitors, the B blasts, do not increase in number and do not express surface Igs. Instead, they give rise to nondividing centrocytes that enter the light zone of germinal centers.[16] It has been assumed that the somatic mutation of Ig V region genes occurs in the centroblasts during this process and that centrocytes are selected on the basis of expressing high-affinity antibodies.

After secondary exposure to antigen, memory B cells might reenter the germinal center via the outer zone of the follicle and undergo another round of hypermutation and clonal proliferation, giving rise to the production of large amounts of high-affinity antibodies.[17] Despite the clear func-tional definition of memory B cells, their phenotype is still discussed controversially. It is generally believed that mem-ory B cells are long-lived cells expressing sIgG and that sIgM+ or sIgD+ cells contribute little or nothing to the mem-ory pool.[1,5,7] However, stathmokinetic considerations con-clude that the fraction of IgG+ cells is by far too low to establish and maintain the memory pool. Because the number of IgG+ cells does not increase with age it has been postu-lated that IgG+ cells can re-express sIgM and sIgD mole-cules.[9,18]

Markers for memory B cells can not be used to identify memory T cells because these markers do not show isotype switching or somatic mutation. A variety of surface mole-cules has been suggested to characterize memory T cells. These cells are proliferating in response to recall antigens and are thought to express high levels of CD2, CD44, CD29, LFA-1, LFA-3, and VLA4-6, and low levels of L-selectin. CD27, and interleukin-2 (IL-2) receptor.[2,6] However, CD45R0, a low-weight isoform of the tyrosine phosphatase CD45, has been suggested repeatedly to be the most promi-nent memory T-cell marker. Interestingly, naive T cells ex-press the high molecular weight isoform of CD45 designated CD45RA.[19,20] In terms of cytokine production memory T cells (CD45R0+) express multiple lymphokines (IL-2, IL-3, IL-4, IL-6, γ interferon [γIFN], and granulocyte-macro-phage colony-stimulating factor [GM-CSF]), whereas naive T cells (CD45RA+) mainly produce IL-2.[21-23]

Classically, G-protein–coupled receptors (GCRs) are acti-vated by hormones or neurotransmitters. However, recent

evidence suggests that this receptor superfamily is also involved in control of immunologic response. The chemokines IL-8 and macrophage inflammatory protein (MIP-1α),[24-26] the anaphylatoxin C5a,[27] and the bacterially derived peptide fMet-Leu-Phe[28] activate leukocytes by binding to GCRs. It has been shown that leukocyte traffic is regulated by binding to specialized high endothelial venules[29] and it has been demonstrated that GCRs play a central role in this multistep process of leukocyte-endothelial interactions.[30,31] We have recently identified a novel member of the emerging family of GCRs in humans and mice. Because it was originally identified in Burkitt's lymphoma cells it was designated BLR1 (Burkitt's lymphoma receptor 1).[32] Northern hybridization and in situ analysis showed that BLR1 expression was limited to certain stages of B-cell development and to distinct areas of the cerebellum.[33,34] We show here that BLR1, a chemokine receptor-related protein, is expressed and regulated on defined lymphocyte subsets. Using a high-affinity monoclonal antibody (MoAb), we demonstrate that this G-protein–coupled receptor is expressed on mature, recirculating B cells and defines a subpopulation of T-helper memory cells.

MATERIALS AND METHODS

Antibodies. The following antibodies were used in this study: anti–CD3-phycoerythrin (PE), anti–CD4-fluorescein isothiocyanate (FITC), anti–CD8-PE and -FITC, anti–CD14-PE, anti–CD19-FITC, anti–CD25-PE, anti–CD45-FITC, anti–HLA-DR-FITC, anti–Leu8-PE, and anti–IgM were all purchased from Becton Dickinson (Mountain View, CA). Anti–CD10-FITC, anti–CD20, anti–CD24, anti–CD29-FITC, anti–CD37, anti–CD38-FITC, anti–CD39, anti–CD45RA-FITC, anti–CD45RO-PE, anti–CD72, anti–CD77, goat-antirat (Fab)$_2$-Peroxidase (POD) or -FITC, mouse-anti-rat-biotin, rat-antimouse-biotin were purchased from Dianova (Hamburg, Germany). Anti–CD5-PE, anti–CD19-PE, anti–CD44-PE, and anti–IgD-Biotin were obtained from Sigma (St Louis, MO). Anti–CD40 was purchased from Serotec (Oxford, UK) and anti–IgG-FITC was purchased from Dakopatts (Hamburg, Germany). Anti–CD76 (clone HD66) was a gift from Dr Bernd Dörken (Max-Delbrück-Centrum, Berlin-Buch, Germany) and anti–CD3 (clone 26-II-8; rat IgG$_{2b}$) was kindly provided by Dr Rolf Schuh (Fresenius, Munich, Germany). All superparamagnetic-labeled antibodies used were purchased from Miltenyi (Bergisch Gladbach, Germany). The anti-BLR1 MoAb RF8B2 (rat IgG$_{2b}$, binding constant [k$_B$] = 2 × 10^{10} mol/L) was produced at our laboratory and is characterized elsewhere[35,36] (Emrich et al, Cell Mol Biol, in press). In brief, Lou/C rats were immunized with 2 × 10^7 live human embryonic kidney 293 cells transfected with BLR1 encoding cDNA. The animals were boosted once with the same amount of 293-BLR1 cells. Spleen cells of the rats were fused with X63Ag8.653 cells and 7 to 9 days later hybridomas were tested for BLR1-specific antibodies by differential screening on 293-BLR1 transfected cells versus untreated 293 cells. Using the anti-BLR1 MoAb RF8B2, Western analysis showed that BLR1 is a glycosylated membrane protein with an apparent molecular mass of about 60 kD[36] (Emrich et al, Cell Mol Biol, in press).

Cell preparations. Peripheral blood lymphocytes (PBLs) were isolated from citrated venous blood from healthy donors. Mononuclear cells were separated by centrifugation on Ficoll gradients (Biochrom, Berlin, Germany) for 30 minutes at 1,200g. The interphase was harvested and cells were washed twice in staining buffer (phosphate-buffered saline [PBS], 4% fetal calf serum [FCS], 5 mmol/L EDTA, 0.1% NaN$_3$, pH 7.4) at 4°C and kept on ice until further

use. Cord blood lymphocytes, obtained from newborns after regular vaginal delivery, were isolated by the same procedure. To identify BLR1 expression on hematopoietic cells, normal sternal bone marrow (BM) cells were obtained from adults undergoing median sternotomy and cardiovascular surgery. BM cells from a patient suffering from plasmacytoma were obtained from BM aspirates and were isolated by lysis of contaminating red blood cells (RBCs) using the NH$_4$Cl method.

Normal tonsil tissues were obtained from tonsillectomies at Munich University Hospital. Tonsil tissues were minced and strained through a stainless steel mesh. To remove debris, cells were centrifuged over a Ficoll cushion as described above. CD19$^+$ B cells were further enriched, as described previously, using the MACS system (Miltenyi).[37] In brief, 8 × 10^7 tonsillar lymphocytes were incubated with an anti–CD19-FITC antibody at 12°C for 15 minutes. After washing once in PBS, 20 μL of a superparamagnetic-labeled rat-antimouse IgG$_1$ antibody was added and the total volume was adjusted to 200 μL. After 15 minutes, cells were washed again and positively enriched on an undersized MACS column with a capacity for 3 × 10^7 cells. The resulting cells were typically greater than 98% CD19$^+$ and less than 1% CD3$^+$, as determined by flow cytometry.

Thymic tissue was obtained from children 3 to 5 years of age undergoing cardiovascular surgery at the Deutsches Herzzentrum (Munich, Germany). Suspensions were made as described for tonsillar lymphocytes, but omitting the Ficoll separation step. One part of the suspension was enriched for CD3$^-$CD4$^-$CD8$^-$ cells by incubating 2 × 10^7 thymocytes with saturating amounts of anti–CD3, anti–CD4, and anti–CD8 MoAb for 15 minutes. After one wash, cells were incubated with superparamagnetic-labeled rat-antimouse-IgG$_1$ and labeled cells were removed by MACS, using an oversized column (capacity, 10^8 cells). Cells of the flow-through were collected and analyzed by flow cytometry.

Cell culture. B cells were cultured in Iscove's medium supplemented with 5 μg/mL bovine insulin, 0.5% bovine serum albumin (BSA), 50 μg/mL human transferin, 5 × 10^{-5} mol/L β-mercaptoethanol (β-ME) (all from Sigma), and 5% IgG-free FCS (PAA, Linz, Austria), as described elsewhere.[38] All cultures were performed in the presence of irradiated (8,000 rad) CDw32-transfected Ltk$^-$ cells (CDw32 L cells), kindly provided by K. Moore (DNAX, Palo Alto, CA). CDw32 L cells were seeded in 48-well flat-bottom microtiter plates at 2 × 10^4/well 1 day before MACS-separated CD19$^+$ B cells were added at 5 × 10^5/well. At the beginning of the experiment, 100 U/mL recombinant human interleukin-4 (rhuIL-4; Genzyme, Cambridge, MA) and 1 μg/mL anti–CD40 MoAb (clone B-B20) were added to give a final volume of 500 μL. Half of the supplemented medium was replaced every other day. BLR1 expression of triplicate cultures of B cells was monitored over a 10-day period by flow cytometry. T cells were cultured in RPMI 1640 medium supplemented with 10% FCS and 5 × 10^{-5} mol/L β-ME. For T-cell activation, 24-well plates were coated overnight with anti–CD3 MoAb (clone 26-II-8; 50 μg/mL in carbonate buffer, pH 9.6). After washing extensively with PBS, PBLs were added at 2.5 × 10^5/well and BLR1 expression of CD3$^+$ cells was monitored as described for B cells.

Immunohistochemistry. Double-immunoenzymatic labeling was performed in accordance to standard techniques.[39] Tissue specimens were kept in buffered isotonic solution at 4°C for less than 4 hours until they were snap-frozen in chilled isopenten or directly embedded in tissue tek (Miles, Elkhart, IN), frozen on a mixture of dry ice and ethanol, and stored at −80°C. Acetone-fixed cryostat sections of 10 μm were incubated in 0.3% H$_2$O$_2$ in PBS for 10 minutes at room temperature (RT) to block endogenous peroxidases. Sections were incubated with the rat anti-BLR1 antibody (10 μg/mL) at RT in a humidified chamber for 1 hour. After three washes in PBS 0.1% Tween 20, a polyclonal goat-antirat F(ab)$_2$ peroxidase conjugate was

added (1:100). After 1 hour, the reaction was visualized using DAB (Sigma). To saturate the goat-antirat conjugate, sections were blocked with 10% rat serum for 15 minutes and all subsequent steps were performed in the presence of 10% rat serum. Slides were then incubated with the second primary murine MoAb (1:40 for 1 hour at RT) and, after three washes in Tris-NaCl (pH 7.4), a rat-anti-mouse-biotin antibody was used (1:100 for 1 hour at RT). The third layer was a streptavidin-alkaline phosphatase (AP) conjugate (GIBCO, Grand Island, NY; 1:80 for 45 minutes). After three final rinses, the AP was developed using Fast Red or Fast Blue (Sigma). In single immunoenzymatic labeling experiments using DAB, the bright brown color of the dye was intensified using $CuSO_4$ (0.5% in isotonic saline solution; 2 minutes at RT) before sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted in Polymount (Polysciences, Eppelheim, Germany). Photographs were taken with a Leica camera (Bensheim, Germany) or Kappa video camera (CF15/2; Göttingeu, Germany). Digitized images (Photo-CD; Kodak, Munich, Germany) were printed on a Mitsubishi video printer (CP100; Ratingeu, Germany) using a control software for high resolution output (Metzger, Munich, Germany).

Flow cytometry. For triple-fluorescence analysis, cells were incubated with biotinylated anti-BLR1 MoAb (5 μg/mL) in PBS (4% FCS, 5 mmol/L EDTA, 0.1% NaN_3) for 20 minutes at 12°C. Cells were then stained simultaneously with streptavidin-cychrome (1:200; Pharmingen, San Diego, CA) and further FITC- and/or PE-conjugated primary antibodies (5 μg/mL). In experiments using unconjugated antibodies, cells were incubated with purified antibody and then stained with F(ab)₂ FITC-conjugated antispecies IgG and IgM. Free binding groups of the secondary antibody were blocked by incubating the cells with 10% serum of the same species from which the primary antibody derived. Cells were subsequently incubated with biotinylated anti-BLR1 MoAb and staining with streptavidin-cychrome was performed as described above.

RESULTS

Expression of BLR1 on PBLs. The expression of BLR1 on PBLs was examined in nine independent experiments by three-color flow cytometry and the use of B- and T-cell–specific MoAbs. All (range, 99% to 100%) $CD19^+$ peripheral B cells expressed BLR1 (Fig 1A). Interestingly, 15% (6% to 22%) of $CD4^+$ T-helper cells (Fig 1B) displayed this receptor, whereas only 2% (1% to 4%) of $CD8^+$ cytotoxic T cells showed BLR1 expression (Fig 1C). It should be noted that expression levels of this receptor were always higher on PBL B cells than on PBL T cells (Fig 1A and B), and that none of the $CD3^-CD19^-$ double-negative cells express BLR1 (Fig 1D). Interestingly, PBLs expressing BLR1 are generally rather small, as demonstrated by decreased forward scatter intensities (Fig 1E). Neither polymorphonuclear cells (PMN) nor monocytes express any detectable levels of BLR1 (data not shown).

Memory T-cell phenotype of $BLR1^+$ T cells. The presence or absence of certain isoforms of the common leukocyte antigen CD45 is thought to be a marker for different stages of T-cell activation and differentiation. It has been suggested that CD45RA is mainly expressed on naive T cells, whereas activated and memory T cells express the CD45R0 isoform.[19,20] Both PBLs and tonsillar lymphocytes were therefore stained with anti–CD45RA-FITC, anti–CD45R0-PE, and anti–BLR1-biotin/streptavidin-cychrome. Setting gates on $BLR1^+CD4^+$ cells showed that about 90% to 97% of these cells of both sources expressed CD45R0 (Fig 2A) and
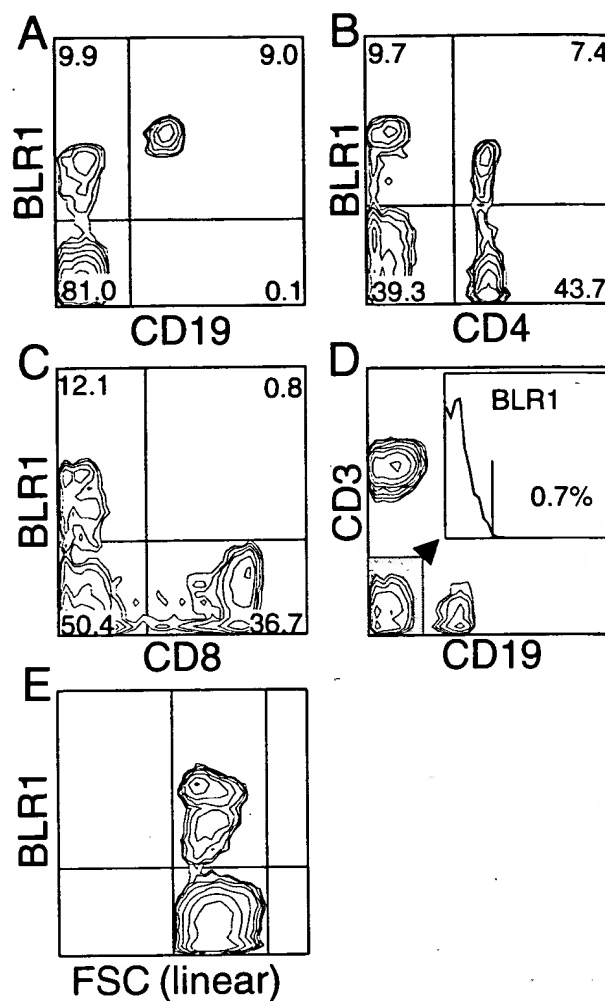
Fig 1. Three-color flow-cytometric analysis of PBLs. Lymphocytes were isolated from peripheral blood and incubated with the BLR1-specific MoAb RF8B2-biotin. Cells were stained with streptavidin-cychrome and incubated with either CD19-FITC/CD3-PE or CD4-FITC/CD8-PE. If not indicated otherwise, the horizontal and vertical scales are log₁₀ fluorescence intensities. Numbers represent the percentage of cells within the corresponding quadrant.

that only 12% of $BLR1^+$ $CD4^+$ cells expressed the CD45RA isoform (data not shown). Furthermore, analysis of activated T-helper cells characterized by the presence of IL-2 receptor (IL-2R) molecules (CD25) illustrates that BLR1 is not expressed on activated T cells (Fig 2B). Interestingly, while analyzing PBLs, BLR1 expression was found on 23% of $CD3^+CD45R0^+$, but on less than 2% of $CD3^+CD45RA^+$ T cells (data not shown). This strongly suggests that BLR1 identifies a subpopulation of memory T cells.

To further investigate the onset of BLR1 expression during T-cell development, thymocyte suspensions were stained with anti–CD4-FITC, anti–CD8-PE, and anti–BLR1-biotin/streptavidin-cychrome. Eight percent of the $CD4^+$ $CD8^-$ cells and 1% of the $CD4^-CD8^+$ cells coexpressed BLR1. However, immature $CD4^+CD8^+$ cells did not express any detectable levels of BLR1 (Fig 3A). To further test the hy-

A

CD4+ CD4+ BLR1+

50% 95% PBL

67% 97% TON

CD45R0

B

CD4+ CD4+ CD25+

16% 0.7% PBL
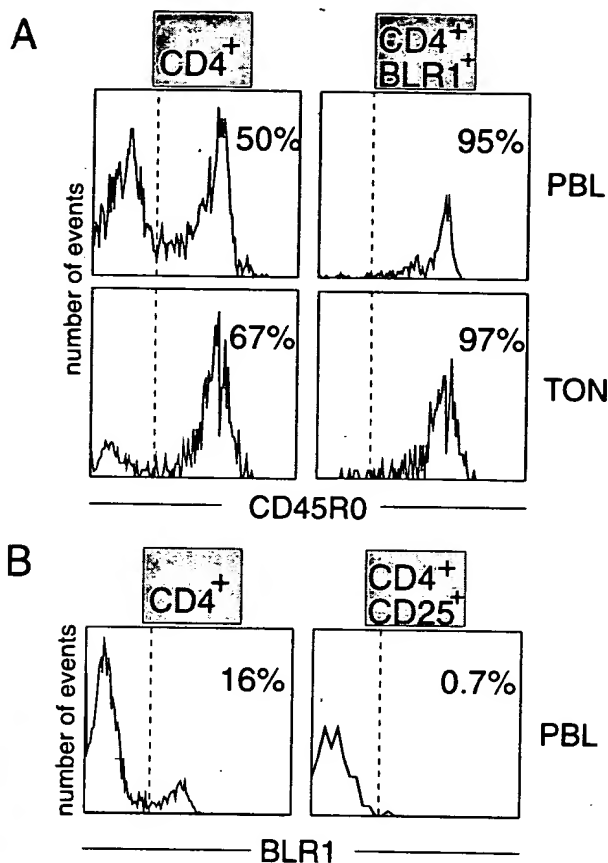
BLR1

Fig 2. Memory phenotype of BLR1+ T-helper cells. Lymphocytes were isolated from peripheral blood (PBL) or tonsils (TON) and were incubated with the BLR1-specific MoAb RF8B2-biotin. Cells were stained with streptavidin-cychrome and incubated with CD4-FITC/CD45R0-PE (A) or CD4-FITC/CD25-PE (B). Gates were either set to all CD4+ cells (CD4+) (A and B) or to CD4+ cells coexpressing BLR1 (CD4+-BLR1+) (A) or to CD4+ cells coexpressing CD25 (CD4+-CD25+) (B). Markers were placed to yield less than 1% positive cells with control antibodies. The horizontal scale is log_{10} fluorescence intensities and the numbers shown are the percentage of positive cells.

pothesis that BLR1 is a marker of antigen-experienced memory T cells, cord blood lymphocytes, which are by definition naive, were tested for BLR1 expression. It was found that greater than 99% of both CD4+ or CD8+ cord blood lymphocytes were BLR1⁻ (Fig 3B). Furthermore, analysis of normal BM cells showed that greater than 92% of CD4+ cells and greater than 98% of CD8+ cells did not express BLR1 (Fig 3C).

These results are contrasted by the high percentage of BLR1+ T cells found in secondary lymphatic tissue, the place
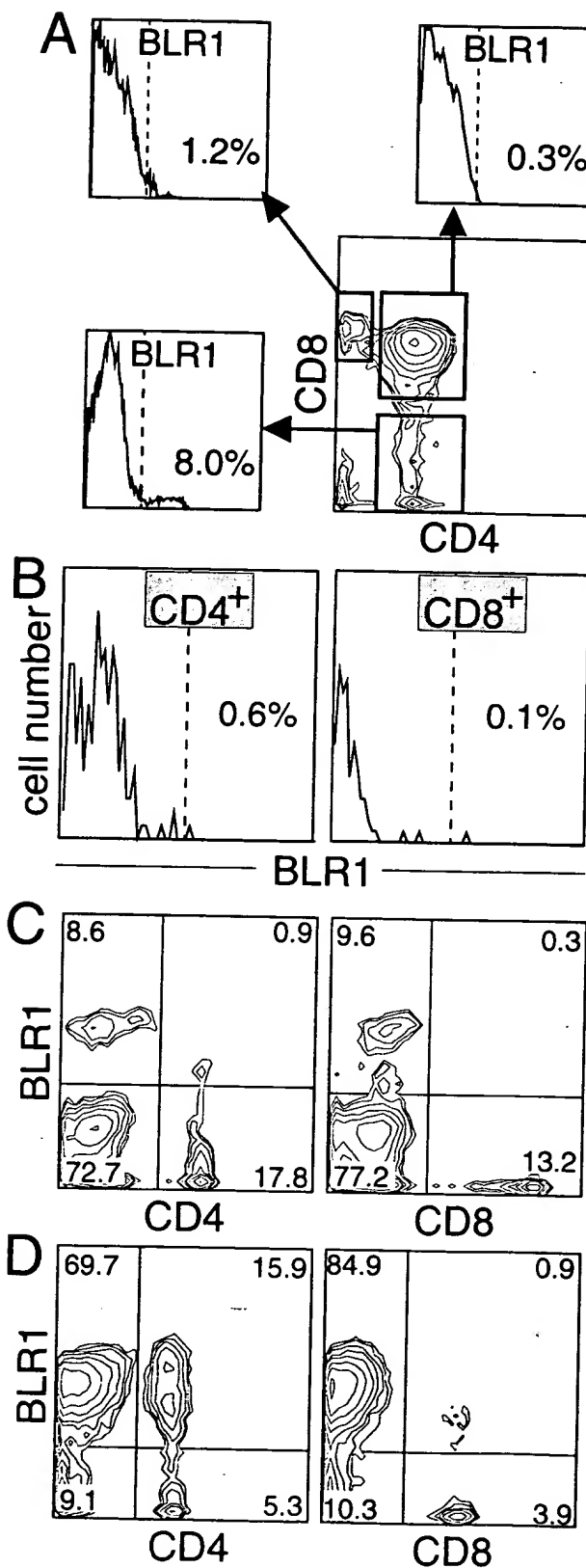
Fig 3. Different expression levels of BLR1 during T-cell development. The BLR1 phenotype of T-cell subsets derived from different lymphatic tissue was determined as described for Fig 1 using BLR1, CD4, and CD8 MoAb. Gates were set on thymocyte subpopulations and the percentage of BLR1+ cells was determined (A). Analysis of expression levels of BLR1 on cord blood cells (B), BM cells (C), or tonsillar lymphocytes (D). Log_{10} fluorescence intensities are shown and the numbers in (C) and (D) represent the percentage of cells within that quadrant.

A

BLR1 1.2%

BLR1 0.3%

BLR1 8.0%

CD8 CD4

B

CD4+ 0.6%

CD8+ 0.1%

BLR1

C

8.6 0.9 9.6 0.3

72.7 17.8 77.2 13.2

CD4 CD8

D

69.7 15.9 84.9 0.9
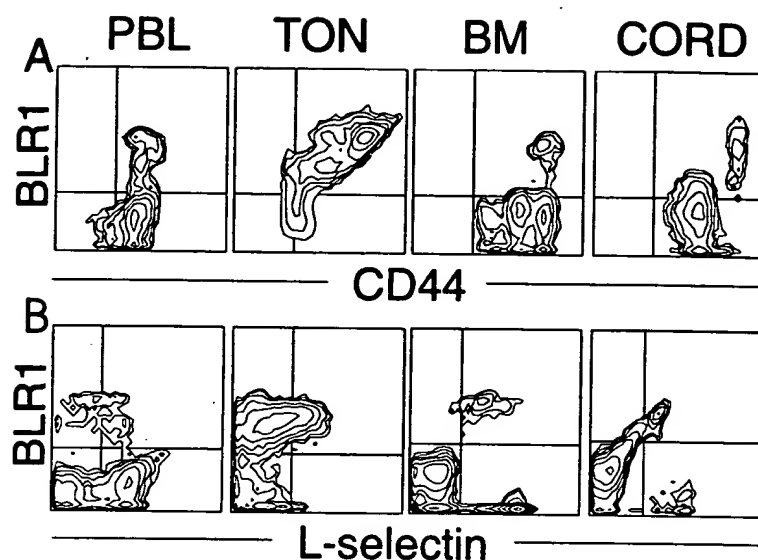
9.1 5.3 10.3 3.9

CD4 CD8

Fig 4. Coexpression of BLR1 and adhesion molecules. Lymphocytes derived from peripheral blood (PBL), tonsils (TON), BM, or cord blood (CORD) were stained with the BLR1-specific MoAb RF8B2-biotin/streptavidin-cychrome and with anti–CD44-PE MoAb (A) or with anti–L-selectin-PE MoAb (B). The horizontal and vertical scales are $\log_{10}$ fluorescence intensities.

where antigen-experienced T-helper cells support B-cell differentiation. Analysis of CD4$^+$ cells in secondary lymphatic organs such as spleen or tonsil showed that 65% of spleen CD4$^+$ cells (data not shown) and 75% of tonsillar CD4$^+$ cells (Fig 3D) are BLR1$^+$, whereas only low percentages for CD8$^+$BLR1$^+$ cells were found (Fig 3D; data for spleen not shown). This finding puts further weight on the hypothesis that the G-protein–coupled receptor BLR1 plays a prominent role in T-cell memory.
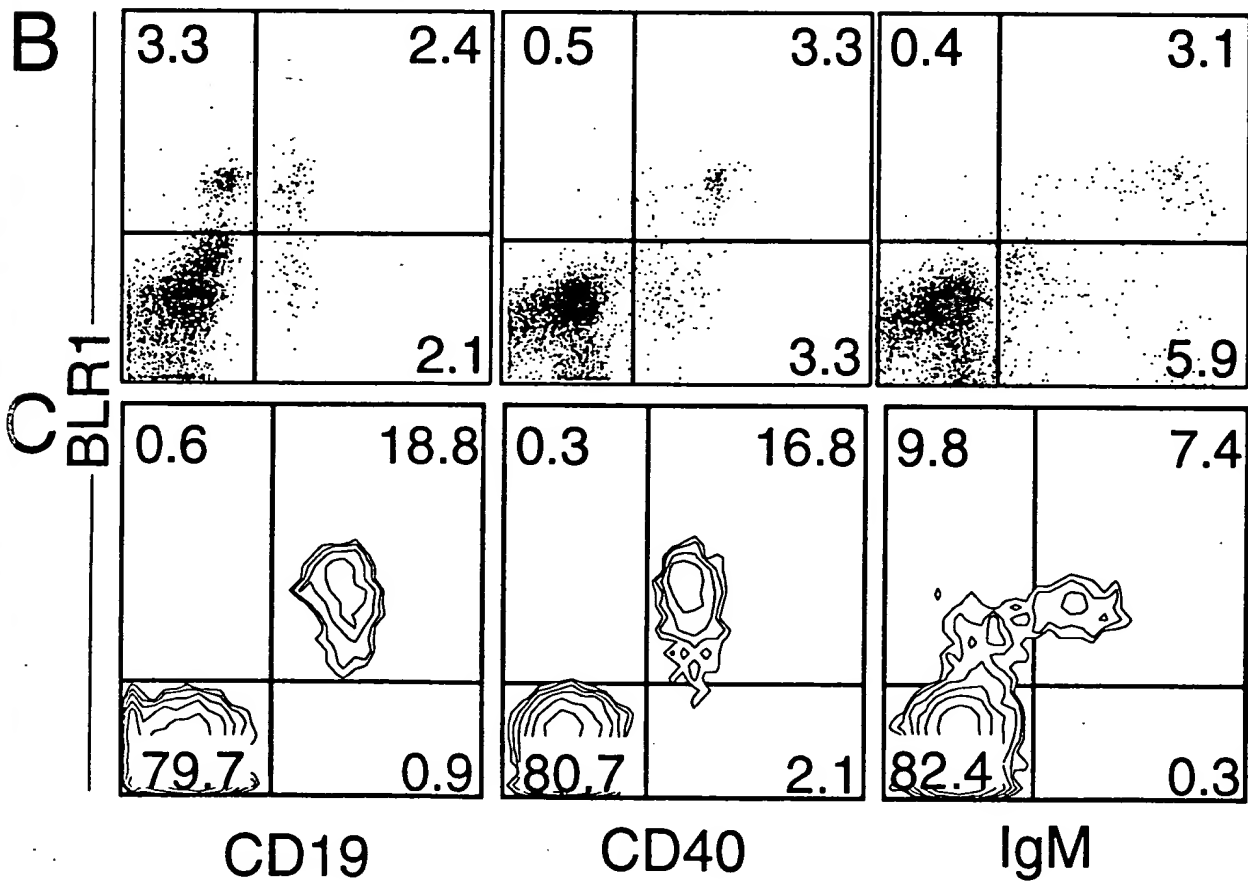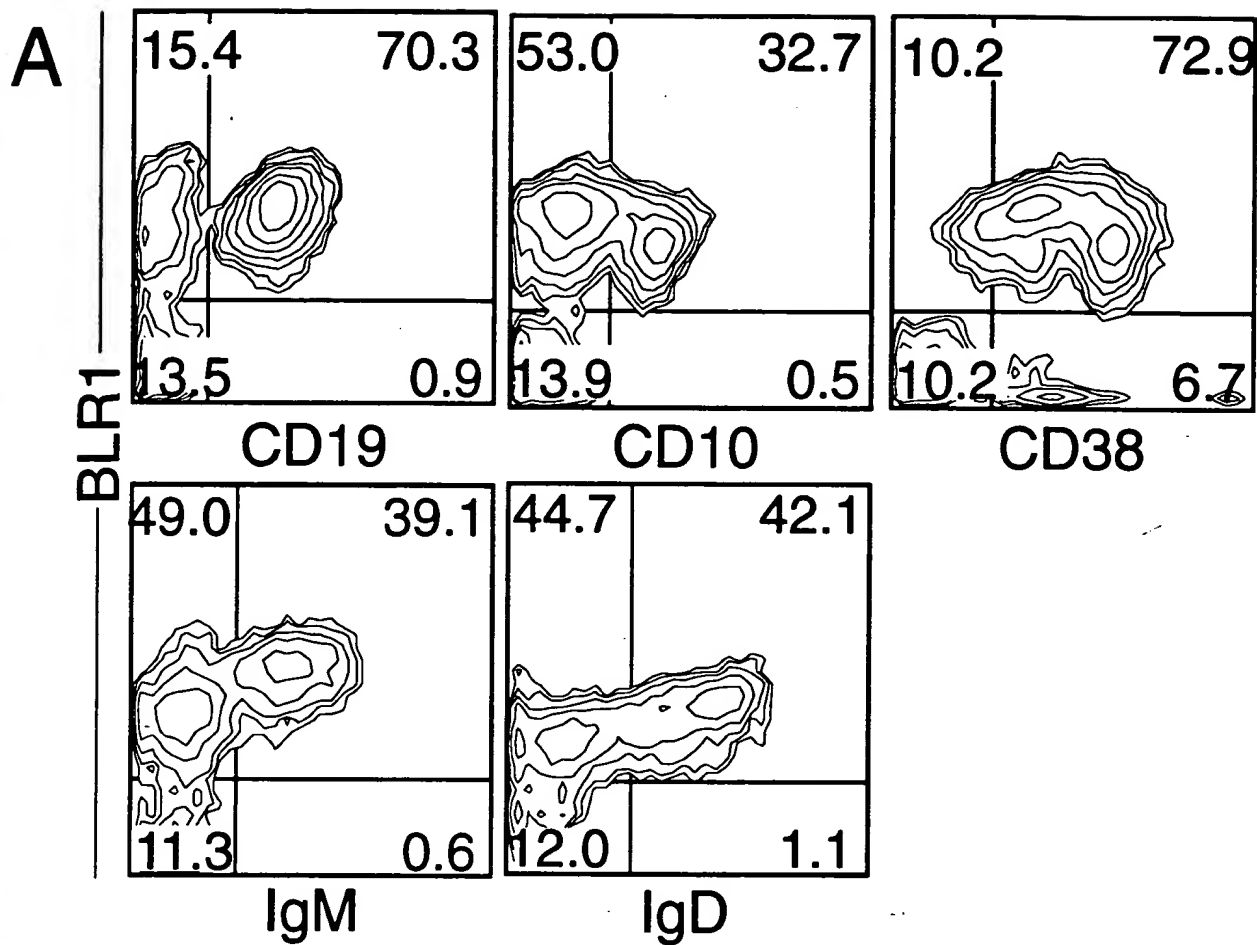
It has been postulated further that long-term memory cells express high levels of CD44,[40] high levels of adhesion molecule VLA-4 (CD29),[41] and low levels of the lymph node homing receptor L-selectin.[42] Therefore, we used appropriated MoAb to stain cells originating from different lymphatic tissues. We found that 50% to 80% of CD3$^+$BLR1$^+$ cells deriving from PBLs or BM express high levels of CD29, whereas only 7% to 15% of CD3$^+$BLR1$^+$ cells originating from tonsils or thymus express this molecule (data not shown). However, all BLR1$^+$ lymphocytes expressed high levels of CD44 irrespectively of their origin (Fig 4A) and, interestingly, BLR1$^+$ cells did not or only moderately expressed L-selectin (Fig 4B). Taken together, these results suggest again that BLR1 is a marker for T-memory cells.

BLR1 identifies mature recirculating B cells. Secondary lymphatic tissues such as spleen, lymph nodes, or tonsils are populated by recirculating B cells and by mature B cells derived from the BM.[16,18] The use of MoAbs directed against surface molecules such as CD19, CD20, CD38, CD40, IgM, and IgD allows the determination of different stages of B-cell maturation. Analysis of BLR1 expression showed that B cells isolated from tonsils were greater than 97% positive for this receptor as demonstrated by coexpression with

CD19, sIgM, or sIgD molecules. High expression levels of BLR1 were always associated with cells positive for IgD, which is a marker for mature B cells (Fig 5A). CD38, a marker of germinal center cells in secondary lymphatic tissues,[43] showed a different correlation with BLR1. Ninety-two percent of CD38$^+$ cells coexpressed BLR1, although cells with high levels of CD38 only moderately expressed this G-protein–coupled receptor (Fig 5A). A similar coexpression pattern was observed for CD10, an antigen thought to be a marker for B blast in secondary lymphatic tissue. More than 95% of CD10$^+$ tonsillar lymphocytes coexpress BLR1, albeit at moderate levels (Fig 5A). The CD77 antigen can be found on a subpopulation of germinal center B cells and is expressed in particular on the centroblasts of the dark zone of the follicle.[44,45] Flow cytometry showed that a high proportion of CD77$^+$ tonsillar lymphocytes express BLR1 (data not shown).

To investigate BLR1 expression at early stages of B-cell maturation, adult BM cells and cord blood were analyzed by three-color flow cytometry. Experiments using BM or cord blood cells showed that only 20% to 60% of each of the CD19$^+$, CD40$^+$, sIgM$^+$, or sIgD$^+$ cells of both sources coexpress BLR1 (Fig 5B, data for BM and sIgD not shown). These data support the notion that this receptor is expressed exclusively on mature recirculating B cells. For a long time the thymus has been considered to be exclusively a place for T-cell development. However, it was recently shown that low numbers of B cells are also present in this organ.[46] To investigate thymic B cells for BLR1 expression, thymocyte suspensions were depleted for CD3$^+$, CD4$^+$, and CD8$^+$ cells by use of MoAbs and MACS. As shown in Fig 5C, virtually all CD19$^+$, CD40$^+$, or IgM$^+$ cells express BLR1. Because

Fig 5. Expression of BLR1 on mature B cells. Tonsillar lymphocytes (A), cord blood lymphocytes (B), or thymocytes depleted of CD3$^-$, CD4$^-$, and CD8$^+$ cells (C) were stained with BLR1- and B-cell-specific MoAb as described in Materials and Methods. The horizontal and vertical scales are $\log_{10}$ fluorescence intensities.

A

BLR1

| 15.4 | 70.3 |
| 13.5 | 0.9 |

CD19

| 53.0 | 32.7 |
| 13.9 | 0.5 |

CD10

| 10.2 | 72.9 |
| 10.2 | 6.7 |

CD38

| 49.0 | 39.1 |
| 11.3 | 0.6 |

IgM

| 44.7 | 42.1 |
| 12.0 | 1.1 |

IgD

B

BLR1

| 3.3 | 2.4 |
| | 2.1 |

| 0.5 | 3.3 |
| | 3.3 |

| 0.4 | 3.1 |
| | 5.9 |

C

BLR1

| 0.6 | 18.8 |
| 79.7 | 0.9 |

CD19

| 0.3 | 16.8 |
| 80.7 | 2.1 |

CD40

| 9.8 | 7.4 |
| 82.4 | 0.3 |

IgM

the BLR1 phenotype of these cells is similar to that of PBLs, the hypothesis is supported that B cells populate the thymus.

Immunohistologic localization of BLR1⁺ cells within lymphatic tissues. To localize BLR1⁺ T and B cells within lymphatic tissues, we took advantage of single- and double-immunohistologic staining techniques. Frozen sections of tonsils were stained with BLR1 MoAb and binding was visualized by DAB, resulting in bright brown color. Binding of a second MoAb (B- or T-cell specific) was shown with the Fast Blue dye. Staining of tonsillar sections showed that the BLR1 MoAb stains both a fraction of follicular and extra follicular cells. However, staining of the antibody was most pronounced in mantle zone cells (Fig 6A). Counterstaining with CD38 MoAb confirmed that there are BLR1⁺ cells within the GC and further demonstrates that only a fraction of CD38⁺ cells express BLR1 (Fig 6B). It has been reported that CD76 also stains mantel zone cells of secondary follicles,[47] but flow cytometry on PBLs showed that BLR1 and the CD76 antigen are different epitopes (data not shown). The use of anti-IgD antibody shows that all cells of the mantle zone coexpress BLR1 and IgD, whereas IgD⁺ cells of the interfollicular zone do not or weakly stain with BLR1 MoAb (Fig 6C). The use of a CD4 MoAb showed that CD4⁺BLR1⁺ cells can be found in the T-cell–rich area between the follicles and also in the germinal center (Fig 6D). Staining of spleen sections shows that BLR1 expression is limited to cells of the periarteriolar lymphatic sheath (PALS) and to singular cells of the red pulp (Fig 6E). Interestingly, cells situated in the centre of PALS express low levels of BLR1 compared with cells of the T-cell–rich area of PALS (Fig 6F).

Downregulation of BLR1 on B and T cells. Functional assays were performed to determine BLR1 expression on CD19⁺ B cells after their differentiation in vitro after ligation of CD40 and the addition of IL-4. For this purpose, 5×10^5 purified B cells were seeded in wells of 48-well trays together with 2×10^4 irradiated CDw32 L cells and anti–CD40 MoAb and IL-4 (100 U/mL). At days 0, 3, 7, and 9 after culture onset, three wells each were harvested and screened for BLR1 expression, using BLR1 MoAb and isotype control MoAb. As shown in Fig 7A, BLR1 was still highly and homogeneously expressed on activated B cells 3 days after culture onset. However, after 7 days, BLR1 could hardly be detected, and was virtually absent after 9 days of culture (Fig 7A). In a similar type of experiment, T cells were activated by anti–CD3 MoAb and expression of BLR1 was monitored over a period of 7 days. As observed for B cells, activation of T cells also led to complete downregulation of BLR1 on these cells (Fig 7B). Despite a transient increase in the number of BLR1⁺ T cells, 3 days after culture onset expression levels of BLR1 were constantly downregulated and the receptor was regularly absent after 5 days (Fig 7B). No changes in BLR1 expression were observed on unstimulated T cells (Fig 7C).

In earlier studies using the Northern blot hybridization technique, we were not able to demonstrate murine BLR1-specific mRNA in plasmacytoma cell lines.[33] To confirm these results, BM cells obtained from a biopsy of a patient with plasmacytoma were analyzed. To allow intracellular

antibody staining, one aliquot of the cells was fixed in paraformaldehyde and subsequently permeabilized using Digitonin. The phenotype of the cell population was examined using (among others) CD10 MoAb, CD38 MoAb, anti-IgG antibodies, and BLR1 MoAb. Surface analysis showed that cells were stained moderately with anti–CD38 MoAb, weakly with both anti–CD10 MoAb and anti-IgG antibody, but not with BLR1 MoAb (Fig 8). In contrast, all permeabilized cells were homogeneously and brightly stained for CD10, CD38, and IgG, but these cells stained not specifically with BLR1 MoAb (Fig 8). Taken together, these results clearly indicate that BLR1 is expressed on mature B cells and expression is downregulated when terminal differentiation to IgG-secreting cells is initiated.

DISCUSSION

This report describes the expression and regulation of BLR1, a heterotrimeric G-protein–coupled receptor sharing significant homology with members of the chemokine receptor family and with neuropeptide receptors.[32-34] The use of a high-affinity BLR1-specific MoAb ($k_B = 2 \times 10^{10}$ mol/L) demonstrates that the expression of this receptor is confined to certain stages of B-cell development and to defined subpopulations of T cells. Using PBLs and three-color flow cytometry, BLR1 can be identified on approximately 15% of the CD4⁺ T-helper cells and on approximately 2% of CD8⁺ cytotoxic T cells. Interestingly, BLR1⁺CD4⁺ cells have the phenotype of resting T-memory cells because they express the CD45R0 isoform and have low to moderate levels of L-selectin, but high levels of CD44, and do not express receptors for IL-2.[2,19,20,40,42] Furthermore, analysis of secondary lymphatic tissue, the site at which T-cell–dependent B-cell development takes place, showed that the majority (60% to 80%) of T-helper cells do express BLR1. Immunohistochemistry shows that those cells have close contact to circulating and eventually maturing B cells. The finding that antigen-inexperienced T cells were derived from cord blood and the fact that immature CD4⁺CD8⁺ thymocytes do not show BLR1 expression further support the idea of the memory phenotype of CD4⁺ cells expressing BLR1.

As with T cells, the expression of BLR1 is also limited to certain stages of B-cell maturation. The CD19 molecule is expressed on all B cells throughout their development, but not on plasma cells.[45] After rearrangement of heavy chain genes, some B cells express surface IgM and are thus defined as immature B cells. Analysis of B cells derived from BM and cord blood showed that only about half of the CD19⁻ B cells express BLR1. Furthermore, because there are still sIgM⁺ B cells in these organs that do not express BLR1, it seems likely that this GCR is first expressed on mature B cells and is present on all recirculating peripheral sIgM⁺ or CD19⁺ B cells. Flow cytometry and immunohistochemistry of tonsillar lymphocytes showed that BLR1 is expressed at high levels on mature resting B cells of the mantle zone, but expression levels are low on germinal center B cells expressing high levels of CD10 or CD38 molecules. This finding suggests that BLR1 is gradually downregulated during affinity maturation.

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Fig 6.
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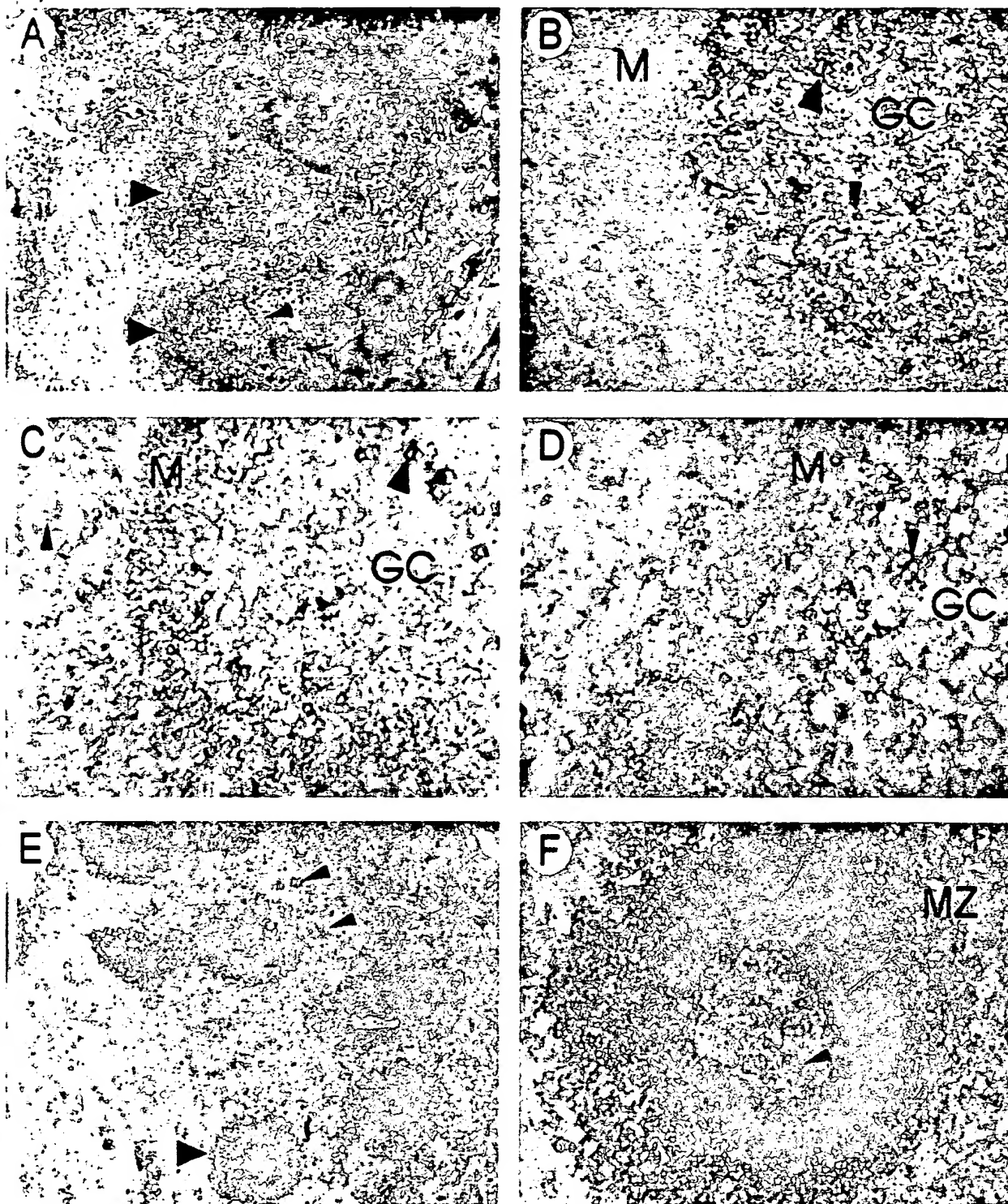dentritic
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Fig 6. Immunohistochemical staining of tonsillar (A through D) and spleen (E and F) sections. Cryostat sections were immunostained with BLR1 MoAb and DAB and counterstained with hematoxylin showing staining of the mantel zone (large arrows in A) and of individual germinal center cells (small arrows in A). Double immunostaining was performed using BLR1 MoAb and the peroxidase-sensitive dye DAB (brown color) and different primary antibody and the AP-sensitive dye Fast Blue (blue color). Cells binding both antibodies stained green (B through D). The use of CD38 MoAb shows that some of the centrocytes coexpress BLR1 (small arrows in B), whereas others only heavily stain for CD38 (large arrows in B). Staining with anti-IgD antibodies shows that most of the BLR1⁺ germinal centre cells do not express IgD, whereas some follicular dendritic cells heavily stain with anti-IgD antibodies (large arrow in C). Most IgD⁺ cells of the interfollicular area do not express BLR1 (small arrow in C). Counterstaining with CD4 MoAb shows that T-helper cells in the follicle coexpress the G-protein–coupled receptor (arrow in D). Immunohistochemical analysis of spleen sections was performed using BLR1 MoAb and the Fast Red dye followed by counterstaining with hematoxylin (E and F). Staining of singular cells of the red pulp (small arrows in E) as well as strong staining of the PALS lymphocytes (large arrows in E) can be seen. Lymphocytes situated in the centre of the PALS only expressed low levels of BLR1 (arrow in F). GC, germinal center; M, mantel zone; MZ, marginal zone (approximate original magnifications: A and E, ×25; B, ×175; C and D, ×300; F, ×250)
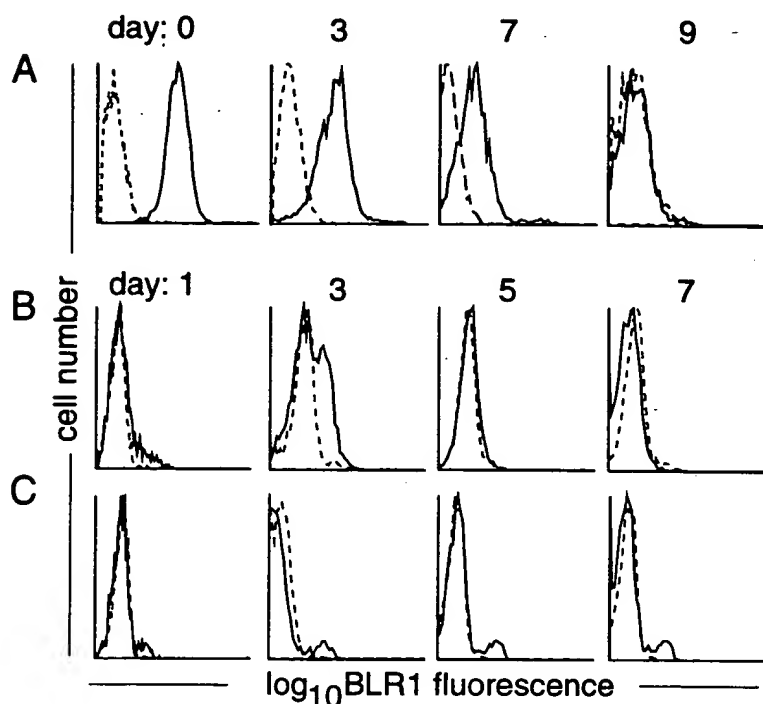
Fig 7. Downregulation of BLR1 on activated B and T cells. CD19⁺ tonsillar lymphocytes were isolated by MACS as described in Materials and Methods. Cells were cultured on irradiated mouse fibroblasts transfected with the human Fcγ-receptor in the presence of anti–CD40 MoAb and IL-4 (A). PBLs were cultured in the precence (B) or absence (C) of pre-coated anti–CD3 MoAbs. At the times indicated, samples were analyzed by flow cytometry for the expression of BLR1 using the biotinylated RF8B2 MoAb (solid lines in A). For the analysis of activated T cells, gates were set to CD3⁺ cells and BLR1 expression was determined using FITC-labeled RF8B2 MoAb (solid lines in B and C). In all experiments, isotype controls are depicted as dashed lines.

with IL-4, MoAbs to CD40, and a mouse fibroblast cell line transfected with human Fcγ receptors results in B-cell proliferation and secretion of Igs.[48] Using this CD40 system, we were able to show that expression levels of BLR1 decrease during B-cell activation. Because none of the cells cultured for 9 days retained detectable levels of BLR1, it seems that triggering of B cells with IL-4 and anti–CD40 MoAb is sufficient to induce downregulation of BLR1. The proposal that BLR1 expression ceases during terminal differentiation of B cells towards IgG secreting plasma cells was based on earlier studies because we failed to demonstrate BLR1-specific RNA transcripts in various murine plasmacytoma cell lines.[33] This result was confirmed in the present study because we did not find BLR1 molecules on human primary plasmacytoma cells. Interestingly, BLR1 is not ex-

pressed on activated (CD25⁺) peripheral blood T-helper cells (Fig 2B), and activation of resting T cells with anti–CD3 MoAb leads to complete downregulation within 5 days. This finding further suggests that BLR1 is mainly expressed on resting lymphocytes.

Several families of cell adhesion molecules, including selectins and integrins, are involved in leukocyte trafficking into secondary lymphatic organs and into sites of inflammation.[49,50] However, mechanisms controlling targeting of specific lymphocyte effector subpopulations during immune challenge are hardly understood. Recent evidence suggests that members of the chemokine family are involved in this process. It has been demonstrated that IL-8 is a chemoattractant for T cells[51] and it has been postulated that RANTES attracts T cells of the memory phenotype.[52] Macrophage

Fig 8. Absence of BLR1 expression on plasmacytomas. Lymphocytes were isolated from a BM aspirate of a patient with plasmacytoma. Cells, either untreated (SUR) or permeabilized (PER), were incubated with the antibodies indicated (——) or isotype control antibodies (---).
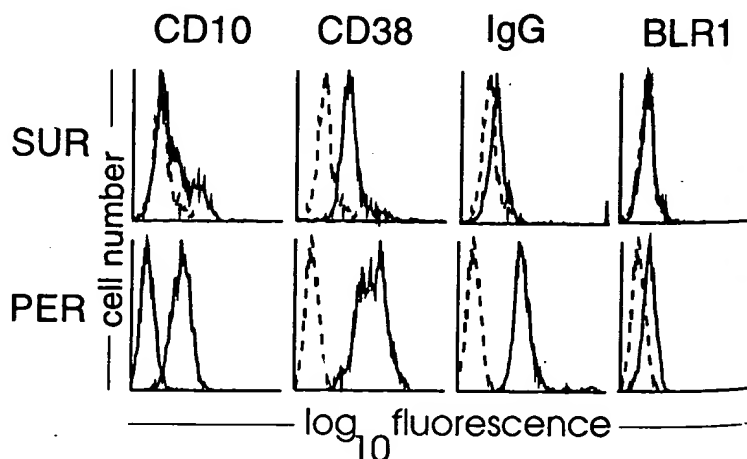
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inflammatory protein MIP-1α attracts both B and T cells, whereas MIP-1β preferentially attracts CD4$^+$ cells. However, others have shown that MIP-1β plays a central role in the recruitment of CD8$^+$ cells from peripheral blood.[31] These investigators propose that this chemokine triggers the conversion of inactive integrins to functionally active molecules and thus selectively directs a defined lymphocyte subpopulation to leave the peripheral blood pool via activation of adhesion molecules.

Several lines of evidence indicate that BLR1 may be involved in similar processes because it fulfills definite criteria. (1) BLR1 shows high homology to receptors belonging to the chemokine family, including IL-8R and MIP-1αR.[24-26] (2) The expression of BLR1 is mainly restricted to defined developmental stages of both recirculating mature B cells and to T memory cells. (3) The number of lymphocytes expressing BLR1 is low in primary but high in secondary lymphatic tissue. These findings raise the possibility that BLR1 is involved in directing the migration of antigen-experienced lymphocytes.

To date, all information about the distribution of leukocyte-specific G-protein–coupled receptors is based on studies of receptor/ligand interactions. The use of a BLR1-specific MoAb allowed us to explore more precisely the expression and regulation of a GCR on lymphatic cells. In addition, BLR1 MoAb may be a valuable tool to identify molecular mechanisms of receptor-coupled signal transduction pathways and may help to identify the physiologic ligand for BLR1.

ACKNOWLEDGMENT

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